Heparan Sulfate as a Mediator of Herpes Simplex Virus Binding to Basement Membrane

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Explants of human lip and oral mucosa were infected with herpes simplex virus (HSV) in vitro and the expression of viral antigen was investigated by immunofluorescent antibody staining. Viral antigen was demonstrated in the cells of basal cell layer and lower prickle cell layers. Moreover, an accumulation of viral antigen in the epithelial-mesenchymal junction was observed. To examine the possibility that the basement membrane has an affinity for HSV, the interaction between HSV and major basement membrane components including type IV collagen, laminin, fibronectin, and heparan sulfate was investigated. When tested by a plaque-reduction assay, only heparan sulfate inhibited HSV plaque formation by competing for the virus adsorption to HEp-2 cells. The inhibitory effects of heparan sulfate and heparin were not affected by pre-incubation of these glycosaminoglycans with antithrombin III, whereas de-N-sulfation resulted in a significant reduction of their inhibitory activity. These findings suggest that heparan sulfate is involved in the binding of HSV to the basement membrane and that N-sulfated glucosamine residues of heparan sulfate are essential for HSV binding. The basement membrane may act as a reservoir of HSV in muco-cutaneous tissues. *J Invest Dermatol 98:494–498, 1992*

Human skin and mucosa are the sites most frequently infected with herpes simplex virus (HSV). It appears that there is a general agreement on the basic features of HSV infection: primary infection is usually initiated at the site of virus inoculation, i.e., muco-cutaneous tissues. Virus replication in the epithelial cells of these tissues results in the development of herpetic lesions such as vesicles and ulcers, followed by a latent infection in neuronal cells at the ganglia. Recurrent lesions will occur if virus from the ganglia moves through nerve axons to peripheral sites and replicates in the epithelial cells [1]. However, details of the pathogenesis of muco-cutaneous HSV lesions is not fully understood. For example, the role of epithelial cell differentiation in the HSV growth at the primary site of infection and the mode of virus spread through the dermal-epidermal junction remain unknown.

Organ cultures are composed of cells at various stages of differentiation and preserve their original structure, providing a proper in vitro system to study the interaction between HSV and muco-cutaneous tissue. To investigate the pathogenesis of mucosal HSV lesions, we have developed an organ culture system with human gingival mucosa explants [2,3]. When these explants were infected with HSV type 1 (HSV-1), viral antigen was expressed predominantly in under-differentiated epithelial cells. Moreover, an accumulation of viral antigen in the epithelial-mesenchymal junction was observed, suggesting the presence of a basement membrane component that has a high affinity for HSV. Type IV collagen, laminin, fibronectin, and heparan sulfate are the major components of the basement membrane [4]. Type IV collagen provides the scaffolding for other components of the basement membrane and promotes adhesion of cultured human keratinocyte [5,6]. Laminin is involved in epithelial cell attachment [7]. Fibronectin is another adhesion factor and is involved primarily in mesenchymal cell attachment [8]. Heparan sulfate interacts specifically with other extracellular matrix components, such as the fibronectin-collagen complex of pericellular matrix [9]. Moreover, it has been shown that heparan sulfate present on the cell surface serves as the initial HSV receptor [10]. However, the roles of the basement membrane and its components in the muco-cutaneous HSV infection have not been studied in detail. In the present study, we show that the association of HSV with the basement membrane can be attributed to heparan sulfate and that N-sulfated glucosamine residues appear to be essential for the interaction between HSV and heparin or heparan sulfate.

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Abbreviations:
bFGF: basic fibroblast growth factor
FITC: fluorescein isothiocyanate
HSV: herpes simplex virus
MEM: minimal essential medium
MOI: multiplicity of infection
PBS: Dulbecco's phosphate-buffered saline
PFU: plaque-forming unit

MATERIALS AND METHODS

Cells and Viruses HEp-2 cells and Vero cells were obtained from Flow Laboratories, Rockville, MA. HEp-2 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum and 2 mM L-glutamine. Vero cells were cultured in MEM containing 5% calf serum and 2 mM L-glutamine. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and air. HSV-1, strain F and HSV type 2 (HSV-2), strain UW-268 were grown in Vero cells [11]. Aliquots were stored at −80°C and used for organ culture studies. For the plaque-reduction assay, virus was partially purified as described below. Vero cells were infected with HSV-1 or HSV-2 at a multiplicity of infection (MOI) of 0.1 plaque-forming units (PFU). After 72 h, the culture media were collected and cen-

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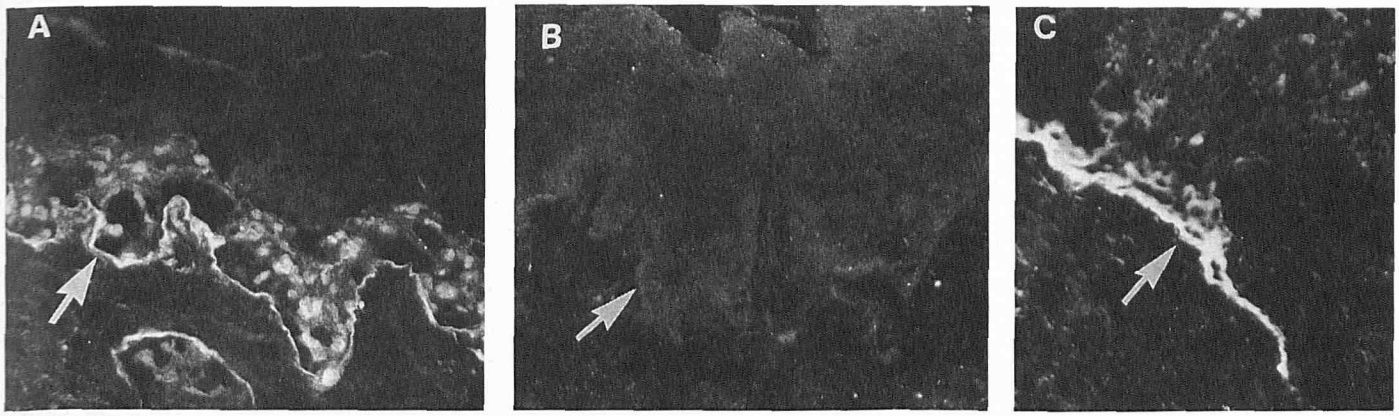

Figure 1. Immunofluorograph of oral mucosal biopsies stained with anti-HSV-1 antibody. In the lip, HSV-1 antigen was expressed in the basal cell layer, lower prickle cell layers, and the basement membrane area, 3 d after infection *(A)*. In the gingiva, HSV antigen was confined to the basal cell layer and the basement membrane area 2 d after infection *(C)*. No specific staining is detected in the mock-infected labial tissue *(B)*. *Arrows,* epithelial-mesenchymal junction. Magnification ×180.

trifuged at 13,000 × *g* for 30 min at 4°C. The supernatant was centrifuged at 110,000 × *g* for 2 h. The resuspended pellet was centrifuged through a 10–30% dextran gradient at 72,000 × *g* for 1 h. The viral peak was collected. Virus was diluted 1:10(vol/vol) in Dulbecco's phosphate-buffered saline (PBS) and centrifuged in a *Centricon-30 concentrator* (Amicon div., W.R. Grace and Co., Danvers, MA) according to the manufacturer's directions to remove most of the dextran.

Organ Culture Normal human gingiva and lip tissues were infected with HSV and cultured as described previously [2,3]. Briefly, tissue fragments were placed in a 24-well tissue plate, covered with 1.5 ml of medium containing 1.5×10^7 PFU of HSV, and incubated for 2 h. Thereafter, tissue fragments were washed in MEM and cultured in an organ culture dish at 35°C. After 2 or 3 d, the cultured tissues were embedded in medium for frozen tissue specimens (O. C. T. compound, Miles Laboratories Co., Elkhart, IN) and stored at −80°C.

Immunofluorescent Antibody Staining Four-micrometer-thick frozen sections from the explants were fixed in acetone at 4°C for 10 min and air-dried. The fixed sections were incubated with polyclonal rabbit anti-HSV-1 antibody (dilution 1:50, Dakopatts, Glostrup, Denmark). After incubation at 37°C for 1 h, the sections were washed with PBS for 30 min and then incubated for a further 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin (dilution 1:40, Dakopatts) with subsequent washing. To determine the location of heparan sulfate in oral mucosa, frozen normal human gingiva sections were fixed in acetone. Thereafter, the sections were reacted with anti-heparan sulfate proteoglycan mouse monoclonal antibody (dilution 1:10, Chemicon International Inc., Temecula, CA) and then FITC-conjugated swine anti-mouse immunoglobulin (dilution 1:100, Dakopatts) as described above. Samples were examined under a Nikon fluorescent microscope.

Plaque-Reduction Assay Engelbreth-Holm-Swarm transplantable mouse tumor-derived laminin was purchased from Collaborative Research, Inc. (Bedford, Inc., Bedford, MA). Bovine plasma derived fibronectin, human placenta-derived type IV collagen, bovine intestinal mucosa-derived heparan sulfate, and porcine intestinal mucosa-derived heparin, which is structurally related to heparan sulfate, were obtained from Sigma Chemical Company (St. Louis, MO). These agents were dissolved in PBS. HEp-2 cells in 24-well plates were inoculated with 200–300 PFU of HSV in PBS containing one of the agents to be tested. After incubation for 30 min, the viral inoculum was removed, the cells were washed twice with PBS, then covered with medium containing 0.3% methylcellulose. After 2 to 3 d of culture at 37°C, plaques were counted. The plaque number formed in cultures infected with HSV in the presence of one of the agents was compared with that in control cultures infected with HSV in the absence of agent. In an experiment, heparin or heparan sulfate was added before virus infection and the cell monolayers were pre-treated for 30 min. Alternatively, one of these agents was added in the methylcellulose medium, after the initial adsorption of the HSV to the HEp-2 cells had occurred. Heparin has high affinity for the protease inhibitor antithrombin III. Binding of heparin to antithrombin III is essential for anticoagulant activity [12]. To test the effect of antithrombin III (Sigma) on the activity of heparin and heparan sulfate, heparin or heparan sulfate was mixed with antithrombin III and incubated for 10 min at room temperature. Thereafter, HEp-2 cells were inoculated simultaneously with the mixture and virus. The infected cells were processed as described above. Results were means of three determinations.

Affinity Chromatography For the preparation of heparin or heparan sulfate-carrying Sepharose 4B beads, 2.8 mg of heparin or 3.1 mg of heparan sulfate was bound to 1 ml of CNBr-activated Sepharose 4B according to the method suggested by the manufacturer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Any remaining active groups were reacted with ethanolamine. The control was Sepharose 4B, of which the active groups were reacted with ethanolamine. One-milliliter columns of heparin Sepharose 4B and heparan sulfate Sepharose 4B were equilibrated with PBS. Virus

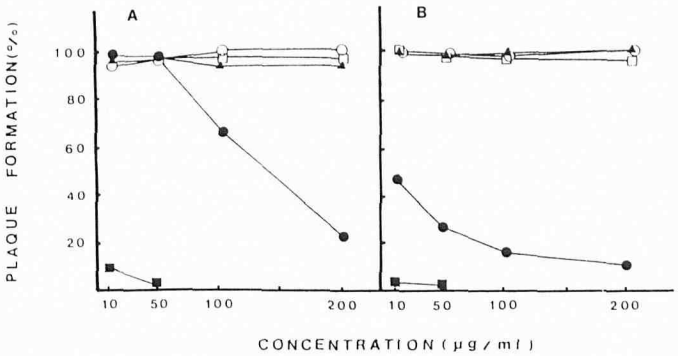

Figure 2. Effect of type IV collagen (▲), laminin (□), fibronectin (○), heparan sulfate (●) and heparin (■) on the plaque formation by HSV-1 *(A)* and HSV-2 *(B)*. Each *point* represents the mean of three determinations.

samples were loaded in 1 ml of PBS, and the columns were washed with 9 ml of PBS. Materials flowing through the columns from loading and washing procedures were pooled and assayed for infectivity.

De-N-Sulfation of Heparin and Heparan Sulfate Heparin and heparan sulfate were de-N-sulfated by the method of Foster et al [13] with a modification as described below. A 1% solution of heparin or heparan sulfate in 0.04 N hydrochloric acid was kept at 95°C for 2 h, cooled, and neutralized with sodium bicarbonate. The samples were dialyzed against running water for 2 d, then dialyzed against saline for a further 2 d. The concentration of these agents was determined by the level of uronic acid in the solutions. Uronic acid was measured by the method of Bitter and Muir [14]. De-N-sulfated heparin prepared by the method of Nagasawa and Inoue [15] was purchased from Sigma.

RESULTS

Demonstration of HSV Antigen in the Basement Membrane Area Viral antigen was demonstrated in the basal cell layer, lower prickle cell layers, and the basement membrane (Fig 1*A*), confirming our previous findings [2,3]. When gingiva explants taken from three individuals were examined 2 d after infection, the area expressing HSV antigen was confined to the basel cell layer and the basement membrane area (Fig 1*C*). Viral antigen was demonstrated in all of the samples infected with HSV-1 but not in the mock-infected labial tissues (Fig 1*B*).

Inhibition of Plaque Formation by Basement Membrane Components When tested at concentrations between 10 and 200 μg/ml, heparin and heparan sulfate significantly reduced the plaque formation of HSV-1, whereas the reduction of plaques by the other agents, including type IV collagen, laminin, and fibronectin, was negligible (Fig 2*A*). In one experiment, virus adsorption was performed at 4°C and a similar result was obtained (data not shown). Heparan sulfate was less efficient in reducing plaque formation as compared with heparin. Under our conditions, the concentrations of heparin and heparan sulfate required to reduce the number of plaque by 50% were 0.7 μg/ml and 150 μg/ml, respectively. The effect of basement membrane components on the plaque formation of HSV-2 was also tested and a similar result to that of HSV-1 was obtained (Fig 2*B*). In this case, HSV-2 was more sensitive than HSV-1 to heparan sulfate. For example, at a concentration of 50 μg/ml heparan sulfate, the plaque number of HSV-1 was reduced to 63% of the control, whereas that of HSV-2 was 20% of the control.

Demonstration of Heparan Sulfate in Human Oral Mucosa The presence of heparan sulfate was demonstrated predominantly in the epithelial-mesenchymal junction and perivascular area of the subepithelial connective tissue (Fig 3).

Treatment of HEp-2 Cell Monolayers or HSV with Glycosaminoglycans Before HSV Infection Pre-treatment of the HEp-2 cell monolayers with either heparin or heparan sulfate had no impact on the plaque formation of HSV-1 (Table I). In contrast, continuous treatment of the HSV-1–infected cells with either of these glycosaminoglycans resulted in a partial reduction of plaque numbers. When HSV-1 was mixed with heparin or heparan sulfate just before infection and cells were treated with these mixtures during the adsorption period, plaque reduction occurred (Fig 2*A* and Table I). However, when 2×10^5 PFU of HSV-1 were incubated with either 10 μg/ml of heparin or 200 μg/ml of heparan sulfate for 10 min and the cells were treated with a thousandfold dilution of this mixture for 30 min, inhibition of HSV plaque formation was no longer observed. This indicates that these glycosaminoglycans did not directly inactivate the viral infectivity.

Affinity Chromatography The titer of unadsorbed virus in the eluate from a Sepharose 4B, heparin–Sepharose 4B, or heparan sulfate–Sepharose 4B column was 4.1×10^4 PFU/ml, 2.4×10^3 PFU/ml and 1.5×10^4 PFU/ml, respectively. The titer of the Sepharose 4B column eluate was approximately 80% of the input virus titer, because of the non-specific decrease of the infectivity during the chromatographic procedure. Based on the assumption that the virus titer that passed through Sepharose 4B corresponded to the net infectious virus applied, it was found that 94% and 63% of the applied viruses were bound to the heparin–Sepharose 4B and heparan sulfate–Sepharose 4B columns, respectively.

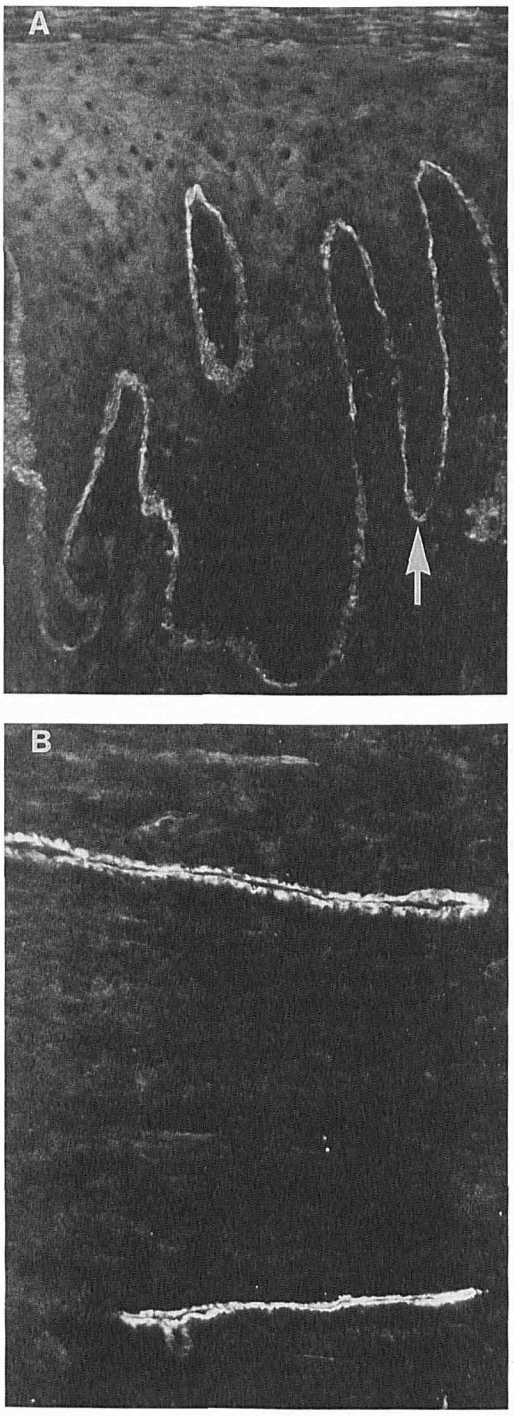

Figure 3. Immunofluorograph of human normal gingiva stained by anti-heparan sulfate antibody. The staining was predominant in the epithelial mesenchymal junction area *(A)* and in the perivascular area of the subepithelial connective tissue *(B)*. *Arrow*, epithelial-mesenchymal junction. Magnification ×180.

Table I. Effect of Glycosaminoglycan Treatment on HSV-1 Plaque Formation

Types of Glycosaminoglycan Treatment	Plaque Formation (% of the control[a])	
	Heparin (10 μg/ml)	Heparan Sulfate (200 μg/ml)
Pre-treatment of cells before virus inoculation	106	107
Pretreatment of virus before inoculation	96	109
Treatment during the virus adsorption period	6	33
Continuous treatment after virus adsorption	47	42

[a] One-hundred percent plaque formation corresponds to the plaque number without drugs.

Table III. Effect of De-N-Sulfated Glycosaminoglycans on HSV-1 Plaque Formation

	Glycosaminoglycan Concentration (μg/ml)	Plaque Formation (% of control)
Experiment 1		
No addition	0	100
Heparin	100	0.7
	10	2
De-N-sulfated heparin	100	87
	10	97
Heparan sulfate	200	36
De-N-sulfated heparan sulfate	200	83
Experiment 2		
No addition	0	100
De-N-sulfated heparin	100	98
	10	99

Effect of Antithrombin III on the Inhibitory Activity of Glycosaminoglycans on HSV Plaque Formation Antithrombin III did not reduce or enhance the effect of these glycosaminoglycans on the plaque formation of HSV-1 (Table II).

Effect of De-N-Sulfated Glycosaminoglycans on HSV Plaque Formation In the presence of 100 μg/ml of heparin or de-N-sulfated heparin during the adsorption period, HSV-1 plaque formation decreased to 0.7% and 87% of the control, respectively. At 200 μg/ml, heparan sulfate reduced the plaque number to 36.2% of the control. When tested after de-N-sulfation, the plaque number was decreased to 82.5% of the control, indicating that the inhibitory activity of heparan sulfate was significantly diminished by de-N-sulfation (Table III, experiment 1). De-N-sulfated heparin prepared by a different procedure was also tested for the effect on plaque formation of HSV. The inhibitory activity of the heparin was also reduced by this alternate method of de-N-sulfation (Table III, experiment 2).

DISCUSSION

In a previous study using the oral mucosa explant cultures, we have shown that HSV-1 or HSV-2 viral antigen was selectively expressed in the under-differentiated cells of the epithelium. Another finding noted in the previous and in the present study was that viral antigen was frequently demonstrated in the basement membrane area. As non-specific binding of the anti-HSV antibody to the basement membrane area was not observed in the sections of mock-infected explants, it was suggested that the basement membrane has a specific affinity for HSV. To investigate this possibility, the interaction of HSV with four major basement membrane components including type IV collagen, laminin, fibronectin, and heparan sulfate was investigated using a plaque-reduction assay. If a substance interacts with HSV or the cellular-binding site for HSV, it will interfere with the adsorption of the virus to cells, resulting in decreased plaque formation [10,16-20]. When tested by this assay, heparan sulfate, but not other components, exerted an inhibitory effect on the plaque formation of HSV-1 and HSV-2, indicating that type IV collagen, laminin, and fibronectin, though they are essential for cell adhesion, are not involved in the association of HSV with the basement membrane.

Table II. Effect of Antithrombin III on the Inhibitory Activity of the Glycosaminoglycans for the Plaque Formation of HSV-1

Concentration			
Heparin (μg/ml)	Heparin Sulfate (μg/ml)	Antithrombin III (unit/ml)	Plaque Formation (% of control)
0	0	0	100
5	0	0	25
5	0	0.1	26
5	0	0.5	26
0	200	0	23
0	200	0.1	22
0	200	0.5	24
0	0	0.5	99

Wudunn and Spear [10] have demonstrated that heparan sulfate is essential for the initial binding of HSV with cells. In their study, however, most experiments were performed using heparin instead of heparan sulfate. Thus, it remained to be established whether heparin and heparan sulfate can interact with HSV in the same manner. In the present study, we demonstrated that heparan sulfate can inhibit plaque formation of both HSV-1 and HSV-2. It was also found that heparan sulfate was less efficient than heparin in the ability to compete for virus adsorption. The concentration of heparan sulfate required to induce 50% reduction of plaque number was 210 times higher than that of heparin. HSV-2 was more sensitive to heparan sulfate than HSV-1.

Pre-treatment of cell monolayers with heparin or heparan sulfate did not affect HSV plaque formation. Moreover, pre-incubation of HSV with either of these glycosaminoglycans, if the mixtures were diluted before inoculation, did not induce any significant reduction of plaque numbers as compared with untreated controls. Thus, heparan sulfate may neither affect the cellular-binding site for HSV nor directly inactivate viral infectivity. We also indicated that HSV-1 bound to a heparan sulfate-Sepharose 4B column, though at a lesser magnitude, as compared to a heparin-Sepharose 4B column. Taken together, it was concluded that heparan sulfate as well as heparin bound virus particles and disturbed the attachment of HSV to the cell surface.

Because the inhibitory activity of heparin and heparan sulfate was not affected by antithrombin III, the binding sites of these glycosaminoglycans for antithrombin III may be different from those for HSV and the binding of antithrombin III does not affect the functional properties of heparin and heparan sulfate.

Nahmias et al [21] examined the effect of synthetic and biologic sulfated polymers on HSV infection and found that the inhibitory potency of the compound depended to a certain extent on the degree of sulfation and on the size of the molecule. In the polysaccharides of heparin and heparan sulfate, L-iduronic acid can be sulfated at the O sites and D-glucosamine can be sulfated at both the O and N sites [22]. It has been shown that weak acid treatment of heparin resulted in de-N-sulfation but that O-sulfate was less sensitive to this treatment [13]. When de-N-sulfated heparin and heparan sulfate were tested for inhibitory activity by the plaque-reduction assay, these de-N-sulfated glycosaminoglycans did not have their original activity levels (Table III), indicating that N-sulfated glucosamine

plays a crucial role in the interaction of HSV with heparin and heparan sulfate. Chondroitin sulfate does not contain N-sulfated saccharide residues [22]. This may in part explain the reason why chondroitin sulfate did not show any inhibitory effect on HSV [21].

Recently, Kaner et al [23] indicated that basic fibroblast growth factor (bFGF) receptor acts as a cellular receptor for HSV-1. Moreover, Baird et al [24] showed that a bFGF like protein is associated with the HSV particle and that this association appears responsible for viral adsorption to the cells. Because bFGF is a heparin-binding growth factor [25], the HSV-associated bFGF can interact with heparan sulfate of the basement membrane. In this regard, bFGF immunoreactivity was detected in muscle basement membranes [26]. As shown in the study of human normal mucosa by immunofluorescent antibody staining (Fig 3), heparan sulfate was present in the basement membrane area of the epithelial-mesenchymal junction and of the perivascular area in the subepithelial connective tissues. The basement membrane may provide a better environment for HSV to bind to heparan sulfate.

Several possibilities exist as to the significance of the association of HSV with the basement membrane. The heparin-bFGF complex protects the growth factor from degradation, thereby enhancing its activity [27]. If bFGF is an essential component for HSV, the binding of virus-associated bFGF to heparan sulfate of the basement membrane will have the advantage for the virus of preserving its infectivity. This means that the basement membrane may act as a reservoir of infectious virus. With respect to recurrent muco-cutaneous infection, reactivated virus particles will be released from nerve ends and concentrated at the basement membrane to ensure infection at the basal cells.

It remains to be determined whether HSV association with the basement membrane observed in the organ culture has any relevance to natural infection in humans, though several reports have suggested the presence of an HSV-associated immunocomplex in the basement membrane area [28–30]. In a more recent study, HSV DNA was detected in the cutaneous lesions of erythemia multiforme using the polymerase chain reaction at a high rate [31]. Thus, the concept that an immune-mediated response directed specifically in the skin is the primary mechanism in herpes-associated erythema multiforme is proposed [31]. If this is the case, HSV should be located primarily within the epidermis [31,32]. HSV and/or immunocomplexes retained in the basement membrane may play a role in the pathogenesis of this particular condition.

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